Biochemical linkage between nitrate-nitrite metabolism and lactate metabolism in oral Veillonella a potential regulatory system to maintain the oral and general health

博士論文

Biochemical linkage between nitrate-nitrite metabolism and lactate metabolism in oral *Veillonella*

– a potential regulatory system to maintain the oral and general health –

口腔 *Veillonella* 属における硝酸／亜硝酸代謝と乳酸代謝間の生化学的連関

―口腔および全身の健康維持のための潜在的調節機構－

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Abstract

[Introduction] *Veillonella* species is one of the major anaerobes in the oral cavity and frequently detected in both caries lesion such as ECC (early childhood caries) and healthy oral microbiome. This bacterium has been known to utilize lactate and to possess the ability to convert nitrate (NO_3^-) into nitrite (NO_2^-). Recently, the interest of NO_3^-/NO_2^- has been increased rapidly, because of its advantageous effects on promoting the oral and general health, by inhibiting the growth and metabolism of oral pathogenic bacteria such as *Streptococcus mutans* and lowering the systematic blood pressure. However, there is only limited information on the regulation of NO_2^- production of *Veillonella* species. Therefore, this study aimed to elucidate suitable environmental conditions for oral *Veillonella* to grow and produce NO_2^-, and their biochemical mechanism by which the NO_2^- production is regulated.

[Materials and Methods] *Veillonella atypica* and *Veillonella parvula* were used as oral *Veillonella* species and *S. mutans* was used as a control. These bacteria were grown under anaerobic conditions, and harvested, washed and resuspended bacteria were used as resting bacterial cells.

[Results and Discussion] There was no effect of NO_3^- on the growth of *S. mutans* and *Veillonella* species, except at high dose (100 mM) on *Streptococcus mutans* ($p<0.05$). The growth of *S. mutans* was inhibited significantly ($p<0.01$) by NO_2^- at a low dose of 0.5 mM, while it needed 20 mM to inhibit the growth of *Veillonella* species ($p<0.01$). Furthermore,

NO_3^-/NO_2^- stimulated the growth of *Veillonella* species by shortening the lag phase of growth. The NO_2^- production of *Veillonella* species was increased by the environmental factors (lactate, acidic pH, and anaerobic condition) and growth conditions (the presence of NO_3^-/NO_2^-), and linked to the anaerobic lactate metabolism. The stoichiometric consideration revealed that NO_3^- was reduced to NO_2^- by accepting the reducing power derived from the oxidization of lactate. These findings suggest that biochemical linkage between NO_3^--NO_2^- and lactate metabolism in oral *Veillonella* species is an important key to maintain the oral and general health.

Keywords : *Veillonella*, nitrate, nitrite, lactate, caries

INTRODUCTION

The oral cavity is an important part of our body, which acts as the first door/ gateway for every substrate to the body. It also plays an important role for mastication, aesthetic, and phonetic, hence maintaining the health in the oral cavity is essential for our health and Quality of Life (QOL). However, the prevalence of oral diseases, notably dental caries in children is relatively high, especially in the developing countries such as Indonesia (Rikesdas, 2014; Achmad *et al*., 2018). Some study shows the prevalence of ECC (early childhood caries) in Jakarta as a capital city of Indonesia is more than 80% over decades. It has been known widely that dental caries is a multifactorial disease. However, one of the main possibilities of high number in caries prevalence in Indonesia is relation with the culture of consuming the sweet food and without be accompanied by appropriate cleaning methods, hence, it would become an issue. Moreover, lack of awareness and poor of knowledge in maintaining the oral health involved in this process (Amalia *et al*., 2019). Since this number is high, many researchers seek the efficient and effective way to suppress it. Then, optimizing beneficial bacteria in the oral cavity through controlling the daily intake of food, drink and snack, as well as promoting the oral hygiene through the oral health education, seem one of the promising options as a caries-preventive strategy to improve this situation.

Veillonella species is known as one of the major oral bacteria and especially detected at high frequency on the tongue surface, buccal mucosa, dental surface, and also have been found in severe early childhood caries (Washio *et al.*, 2005; Mashima *et al.*,

2011). Recently, several *Veillonella* species have been identified in children and healthy young adults, such as *V. atypica, V dispar, V. rogosae, V. tobetsuensis, V. parvula,* and *V. denticariosi* (Mashima *et al*., 2011; Mashima *et al*., 2014). Since lactate is always available in all condition in the oral cavity and *Veillonella* species needs lactate as an energy source, hence *Veillonella* is available both in oral health and oral disease condition. Some study revealed that *Veillonella dispar* was detected mainly in good or moderate oral hygiene in children, while *Veillonella parvula* and *Veillonella tobetsuensis* were mainly detected in the poor oral hygiene group (Nakazawa., 2017). However, their mechanism is still unknown.

As describe above, *Veillonella* species utilize lactic acid as an essential carbon and energy source, then convert it into weaker acids, such as acetic, propionic, and formic (Takahashi., 2015). Dental caries is initiated by the exposure of acid produced by the carbohydrate metabolism of acidogenic microorganisms such as *Streptococcus mutans*, while the acid neutralization such as the conversion of lactic acid to weaker acids can contribute to tilting the balance between demineralization and remineralization of the tooth surface to remineralization (Takahashi, 2015). Therefore, *Veillonella* species has been assumed as a beneficial bacterial species to prevent dental caries.

Besides utilization of lactic acid, *Veillonella* and some oral bacteria possess the ability to produce NO_2^- by reducing NO_3^- (Doel *et al*., 2005). NO_3^- as an essential compound is easily found in the oral cavity, because it can be supplied from green leafy vegetables such as spinach, lettuce and cabbage (Brkić *et al*., 2017). Furthermore, after being consumed and absorbed through the gastrointestinal tract, then approximately 25% of ingested NO_3^- is secreted in saliva (Allaker *et al*., 2001; Fejerskov *et al*., 2015). Therefore,

NO_3^- is always available in the oral cavity. As described above, this NO_3^- can be reduced to NO_2^- by oral bacteria including *Veillonella* species.

NO_2^- has an antimicrobial activity and therefore has been used widely in canned food as a food preservation. In the dental field, NO_2^- is reported to inhibit the acid production of dental plaque (Yamamoto *et al*., 2017), as well as the growth of oral pathogenic bacteria such as *Streptococcus mutans* and *Porphyromonas gingivalis* (Silva-Mendez *et al*., 1999; Allaker *et al*., 2001). Hence, the NO_2^- produced by oral bacteria such as *Veillonella* species might contribute to the prevention of oral diseases such as dental caries and periodontitis. In addition, NO_2^- is known to be able to contribute general health to normalize the blood pressure (Cammack *et al*., 1999, Gilchrist *et al*., 2011). After ingested NO_3^- from daily intake, it comes into contact with symbiotic bacteria in oral cavity and reduces into NO_2^- as describe above, the NO_2^- then enters the circulation and convert into nitric oxide (NO) by mammalian nitrite reductase or acidic stomach, resulting vasodilatation and lowering the blood pressure significantly (Kapil *et al*., 2015, Montenegro *et al*., 2017).

However, there is only limited information on the regulation of NO_2^- production of oral *Veillonella* species. Therefore, the aim of this study was to elucidate suitable environmental conditions for oral *Veillonella* species to grow and produce NO_2^-, and their biochemical mechanism by which the NO_2^- production is regulated.

MATERIALS AND METHODS

1. Bacterial strains and growth conditions

These bacterial type strains, *Veillonella atypica* ATCC 17744 and *Veillonella parvula* ATCC 10740, and *Streptococcus mutans* NCTC 10449 were used in this study. These bacteria were maintained on CDC anaerobe blood agar (Nippon BD, Tokyo, Japan) at 37^{o}C in an anaerobic glove box (N_2, 80%; CO_2, 10%; H_2, 10%; NHC-Type; Hirasawa Works, Tokyo, Japan). *Veillonella* strains were cultured in a complex medium containing 0.5% tryptone (Difco Laboratories, Detroit, MI, USA), 0.3% yeast extract (Difco Laboratories), and 1.26% sodium lactate (Wako, Tokyo, Japan) in 50 mM potassium phosphate buffer (PPB, pH 7) (TYL) under anaerobic conditions in the NHC-type glove box. *Streptococcus mutans* was cultured in a complex medium containing 1.7% tryptone, 0.3% yeast extract, 0.5% NaCl (Wako, Tokyo, Japan), and 0.5% glucose (Wako, Tokyo, Japan) in 50 mM phosphate buffer solution (PPB, pH 7) (TYG) under anaerobic conditions in the NHC-type glove box. All mediums were kept under anaerobic conditions for at least 3 days before use.

2. Effects of nitrate (NO_3^-) nitrite (NO_2^-) on bacterial growth

Bacterial strains were grown in TYL or TYG, with various concentrations (0 - 100 mM) of potassium nitrate (KNO_3) or potassium nitrite (KNO_2) at 37^{o}C for 24 hours under anaerobic conditions. Bacterial growth was estimated by monitoring of the

optical density (OD) of culture medium at 660 nm using spectrophotometer (WPA, Cambridge, UK).

3. Bacterial response to NO_3^- or NO_2^- during growth

Veillonella strains were pre-cultured anaerobically in TYL medium with and without 1 mM sodium nitrate (KNO_3) or 1 mM KNO_2. At the logarithmic phase of growth, these pre-cultured bacteria were transferred to the new TYL medium with or without 1 mM KNO_3 or KNO_2, then, bacterial growth was monitored as described above.

4. NO_2^- production from NO_3^- by the resting cells of *Veillonella* species

Veillonella strains were anaerobically cultured in TYL medium with or without 1 mM KNO_3 or KNO_2. The bacterial cells were harvested at the late logarithmic phase (optical density at 660 nm : 0.8 – 0.9) by using centrifugation (10.000 rpm for 7 min at 4^oC), and then washed twice and re-suspended in washing buffer containing of 75 mM potassium chloride (KCl), 75 mM sodium chloride and 2 mM magnesium chloride in 2 mM PPB (pH 7). These bacterial cell suspensions were stored at 4^oC until use. Bacterial cells were harvested using double-sealed centrifuge tubes to maintain the anaerobic condition. The washing and preservation of the cells were carried out under anaerobic conditions in another anaerobic gloves box (N_2, 90%; H_2, 10%; NH-Type; Hirasawa Works, Tokyo, Japan).

Reaction mixtures containing bacterial cell suspensions (optical density at 660 nm = 1.0), sodium lactate in various concentration (0 – 50 mM) and 1 mM KNO_3 in 40 mM PPB (pH 7 or 5) were prepared. These reaction mixtures were incubated at 37^{o}C in aerobic (in air) or anaerobic conditions for 30 min. After incubation, the reaction mixtures were centrifuged (10,000 rpm for 3 min at 4^{o}C) to obtain the supernatant. The amounts of NO_2^- in the supernatant were measured by using Griess reagent kit (Dojindo, Kumamoto, Japan) (Oyungerel *et al*., 2013; Sasaki et al., 2016) and microplate reader (Thermo Scientific Varioskan Flash, Vantaa, Finland) at 540 nm.

5. Metabolic end products during the NO_2^- production by *Veillonella* species

The bacterial cell suspension grown in TYL medium in aerobic and anaerobic conditions were prepared as mentioned above. The reaction mixtures (1 ml) containing the bacterial cell suspension (the final optical density at 660 nm = 1.0), with or without 1 mM KNO_3 and 10 mM sodium lactate in 40 mM PPB (pH 7 or 5) were incubated for 15 min at 37^{o}C in aerobic or anaerobic conditions. Subsequently, 0.45 mL of the reaction mixture was mixed with 0.05 mL of 6N perchloric acid for the organic acid analysis. The rest of the reaction mixure was centrifuged to remove the cells and the supernatant was stored at 4^{o}C for the NO_2^- analysis. The samples for the organic acid analysis were filtered through polypropylene membrane (pore size: 0.20 μm; Toyo Roshi Ltd., Tokyo, Japan). Then, the filtrates were analyzed by high performance liquid chromatography (HPLC; Shimadzu Prominence LC-20AD,

Shimazu Co., Ltd., Kyoto, Japan) (Manome *et* al., 2019) for the concentrations of various organic acids: pyruvate, malate, succinate, lactate, fumarate, formate, acetate, propionate.

The amount of NO_2^- produced in the sample was also measured by using Griess reagent kit as described above.

6. Statistical analysis

The significance of the differences among multiple groups were analyzed using tukey's test and dunnett's test. P values of <0.05 were considered statistically significant (StatFlex Ver. 6)

RESULTS

1. Effect of NO_3^- or NO_2^- on the bacterial growth

There was no effect of NO_3^- on the growth activity of *Streptococcus mutans* and *Veillonella* species, except at high concentration of NO_3^- (100 mM) on *Streptococcus mutans* (Fig. 1). The growth activity of *Streptococcus mutans* became lower as the NO_2^- concentration in the growth medium was increased (Fig 2). Even in the presence of 0.5 mM NO_2^-, a significant decline was observed compare with control ($p<0.01$), furthermore at over 5.0 mM NO_2^-, the growth was inhibited to less than 12.5%.

Meanwhile, it needed higher concentration of NO_2^- to inhibit the growth of *Veillonella* species, and the growth was not affected even in the presence of 10 mM NO_2^-. The presence of 20 and 100 mM NO_2^- showed a significant decline of the growth were observed ($p<0.01$).

2. Bacterial response to NO_3^- or NO_2^- during the growth

In the presence of 1 mM NO_3^- the length of lag phase was shortened (Fig. 3a and 4a) although the growth rate in logarithmic growth phase and the final OD of growth was not affected. Moreover, this effect was modified by the pre-culture conditions. The lag phase of *V. atypica* in the absence of NO_3^- or NO_2^- became longer when pre-cultured with NO_3^- or NO_2^- and shortened in the presence of 1 mM

NO_3^- or NO_2^- (Fig. 3b and 3c), while that of *V. parvula* became longer only when pre-cultured with NO_3^- (Fig. 4b).

3. NO_2^- production from NO_3^- by the resting cells of *Veillonella* species

The effects of environmental factors (lactate, pH, and atmospheric conditions) and growth conditions (the presence of NO_3^- or NO_2^-) on the NO_2^- production of *Veillonella* species were investigated.

In aerobic condition, both of *Veillonella atypica* and *Veillonella parvula* required lactate to produce NO_2^- and this production was increased obviously at acidic condition (pH 5) by 1.9 – 9.5 and 1.2 – 6.8 times, respectively (Fig 5 and 6). When grown with NO_3^- or NO_2^-, both bacterial strains increased NO_2^- production. In *Veillonella atypica*, the NO_2^- production increased by 4.0 – 263 and 1.5 – 150 times in the presence of NO_3^- and NO_2^-, respectively. In *Veillonella parvula*, the NO_2^- production increased by 3.1 - 56 and 1.1 - 41 times in the presence of NO_3^- and NO_2^-, respectively. Furthermore, the NO_2^- production of *Veillonella atypica* was tended to be higher than *Veillonella parvula*.

In anaerobic condition, the NO_2^- production of *Veillonella atypica* and *Veillonella parvula* (absence of NO_3^- or NO_2^- in the growth medium) was 1.1 – 1.5 times and 1.03 – 4.2 times higher at pH 5 than at pH 7, respectively (Fig. 5 and 6). Similarly to aerobic condition, NO_2^- production increased as lactate concentration increased. Furthermore, the NO_2^- production was 1.7 – 122 times higher than in aerobic condition under all experimental conditions for both *Veillonella* strains, and

the NO_2^- production was detected without the addition of lactate, although the activity was small.

4. Metabolic end products from lactate during the NO_2^- production by the resting cells of *Veillonella* species

Under anaerobic conditions, the metabolic end products from lactate with or without KNO_3 by the resting cells of *Veillonella atypica* were mainly propionate and acetate followed by formate and pyruvate (Fig. 7). The amount of pyruvate was detected at pH 5. Under aerobic conditions, the main end products were pyruvate and acetate with a small amount of propionate. There was no clear difference between at pH 7 and 5. NO_2^- production was observed only in the groups incubated with KNO_3. The total amounts of end product under anaerobic condition was higher than those under aerobic conditions.

The end products from lactate during NO_2^- production by the resting cells of *Veillonella parvula* were mainly acetate with small amounts of pyruvate and propionate in anaerobic conditions (Fig. 8). Small amount of acetate was detected without the addition of KNO_3. In aerobic conditions, the main end product during NO_2^- production was pyruvate and followed by acetate. Without the addition of KNO_3, a significant amount of end product was detected. There was no clear difference between at pH 7 and 5. NO_2^- production was observed only in the groups incubated with KNO_3. The amount of end product under anaerobic condition was higher than aerobic condition.

DISCUSSION

In this study, the growth of *Streptococcus mutans* and *Veillonella* strains were not affected by adding NO_3^-, except after adding NO_3^- at high dose (100 mM) on *Streptococcus mutans* (Fig 1). Furthermore, the growth of *Streptococcus mutans* was inhibited by adding NO_2^- even at a low concentration of 0.5 mM (Fig 2). This result is consistent with the previous report (Silva-Mendez *et al.*, 1999) that NO_2^- was effective to inhibit even stop the growth of *Streptococcus mutans* at its low dose. NO_2^- is reported to interfere the energy metabolism by inhibiting oxygen uptake, oxidative phosphorylation and proton-dependent active transport (Rowe *et al.*, 1979). NO_2^- also causes collapse of the proton gradient, inhibit the metabolic enzymes (Yarbrough *et al.*, 1980), damage the cell membrane and binding the essential protein such as iron-sulfur proteins that play an important role in energy metabolism, and damage DNA after turned to NO by acidified or nitrite reductase (Cammack *et al.*, 1999). On the contrary, the growth of *Veillonella* was tolerant at 10 mM NO_2^- and inhibited by over 20 mM NO_2^- (Fig 2). Logically since *Veillonella* species are nitrite-producing bacteria, they should have a system to tolerate its own production of NO_2^-. However, the tolerant system has not been clarified yet.

The concentrations of NO_3^- and NO_2^- in the oral cavity were reported to be 0.8 mM (unstimulated saliva) – 4 mM (stimulated saliva) and around 0.3 mM, respectively (Sanchez *et al*, 2014). Other study showed that the concentration of NO_2^- was normally found 0.2 – 2 mM (Silva-Mendez *et al*, 1999) and that the concentration of NO_2^- in saliva varies according to dietary NO_3^- intake, activity of bacterial nitrate reductase, salivary flow

rate, and endogenous production of nitrate (Dykhuizen *et al*, 1996). These findings support that NO_3^- itself cannot inhibit the growth activity of *Streptococcus mutans*, however intake of NO_2^- or NO_2^- formed in the oral cavity can inhibit the growth of cariogenic bacteria such as *S. mutans*, but cannot inhibit *Veillonella* species *in vivo*.

NO_3^- even stimulated the growth activity on *Veillonella* species by shortening the lag phase during the growth (Fig 2a and 3a). In the absence of NO_3^- *Veillonella* species seemed to need time to induce some enzymatic systems for entering the log phase of growth; however, growth medium contained NO_3^- seemed to skip this induction. These observations suggest that NO_3^- is tightly linked to the essential physiological properties of *Veillonella* species such as an energy production, although they become able to utilize alternative components contained in the culture media after adaptation.

The duration of the lag phase in the absence of NO_3^- or NO_2^- was extended when pre-cultured with NO_3^- or NO_2^- and shortened in the presence of 1 mM NO_3^- or NO_2^- (Fig. 3b, 3c, and 4b), suggesting that NO_3^-- or NO_2^--precultured *Veillonella* species have already established a physiological system suitable to utilize NO_3^- or NO_2^- and therefore they needed more time to change their system to the system that is not linked with NO_3^- or NO_2^- but with alternative components in the culture media. All these results indicate that *Veillonella* species can adapt flexibly to growth conditions and establish the optimum system for them to enter the logarithmic phase of growth.

The present study clearly showed that *Veillonella* species can produce NO_2^- from NO_3^- and that this NO_2^- production requires lactate (Fig. 5 and 6). Anaerobic activity was higher than aerobic one, suggesting that this metabolic activity is oxygen sensitive,

although an enzyme responsible for NO_3^- reduction was not identified in the present study. The NO_2^- producing activity was higher at acidic condition, similar to *Veillonella*'s production of hydrogen sulfide from cysteine (Washio *et al.*, 2005). In most cases, *Veillonella* species grown with NO_3^- had the highest activity of NO_2^- production (Fig. 5 and 6), indicating that NO_3^--grown cells have an established system to utilize NO_3^- efficiently, as discussed above (Fig. 3 and 4). NO_2^- also had similar effect in some cases (Fig. 5 and 6).

Analyses of amounts of end products from lactate and NO_2^- production from NO_3^- revealed a linkage between lactate metabolism and NO_3^- reduction. In *Veillonella parvula*, very small amount of end product was found under anaerobic conditions without adding NO_3^-, while significant amount of end products was detected by adding NO_3^- along with a significant NO_2^- production (Fig.8). This observation showed a tight linkage between lactate metabolism and NO_2^- production. On the other hand, under aerobic conditions, a significant amount of end products was detected with or without adding NO_3^- along with small amount of NO_2^- production in the group with adding NO_3^-, indicating a weak linkage between lactate metabolism and NO_2- production. These results are consistent with the previous report by Hoshino *et al.*, (1981) that *Veillonella alcalesence* utilized lactate into acetate and propionate under aerobic conditions. These observations suggest that oxygen may function as an electron acceptor in the lactate oxidation (metabolism) for a smooth metabolism of lactate under aerobic conditions. On the other hand, there was no clear difference of end product with or without NO_3^- under anaerobic conditions in *Veillonella atypica*. Similarly, this condition was also observed under aerobic condition (Fig. 7). This

observation showed a weak linkage between lactate metabolism and NO_2^- production in *Veillonella atypica*.

The difference in tightness of linkage between lactate metabolism and NO_3^- reduction among *Veillonella* species is probably due to the bacterial species specific characteristic. This result suggests that *Veillonella atypica* is able to utilize lactate by using unknown electron acceptor instead of NO_3^- under anaerobic conditions, possibly hydrogen ion ($2H^+ + 2e^- \rightarrow H_2$) in the catalysis of hydrogenase that was found in *Veillonella* species (Valentine and Wolfe, 1963).

The stochiometric consideration of metabolic end product supports the metabolic linkage between lactate metabolism and NO_2^- production more clearly especially for *Veillonella parvula*. Under anaerobic conditions, *Veillonella* species produced propionate, acetate, formate and pyruvate with the production of NO_2^- from NO_3^- (Fig. 7 and 8), where lactate could be oxidized to pyruvate and NO_3^- could be reduced to NO_2^- (Fig. 9). Produced pyruvate could be further metabolized to formate, acetate and propionate through the formate-acetate pathway and the propionate pathway depending on the reduction-oxidation balance. In other words, according to the stoichiometric calculation of metabolic pathways (Fig. 9), if L mM lactate is utilized and N mM NO_2^- is produced, L-N mM of reducing power has to be used in the following three pathways ((i) pyruvate → formate + acetate; (ii) pyruvate → acetate + 2H; (iii) pyruvate + 4H → propionate). The amounts of pyruvate (pyr mM) propionate (pro mM), acetate (a mM) and formate (f mM) are calculated to satisfy the following equation: 2a+pyr-pro-f = N, where L = pyr+a+pro [for carbon recovery] and (L-N)+(a-f) = 2p [for redox recovery]. This calculation fits well for the results of end products

of anaerobic metabolism. These metabolic properties clearly show the mutual dependency of anaerobic lactate metabolism with NO_3^- reduction in *Veillonella parvula* since lactate was not metabolized without NO_3^- (Fig. 8). As discussed above, *Veillonella atypica* might utilize an alternative electron acceptor instead of NO_3^- under anaerobic conditions and this explains why this stoichiometric calculation does not fit well in *Veillonella atypica*.

Furthermore, the difference in the end product profile between *Veillonella* species (Fig. 7 and 8) could be due to the balance between the activity of the formate-acetate pathway and the propionate pathway. The propionate pathway of *V. atypica* seems to be more active than that of *V. parvula*.

Under aerobic conditions, acetate and pyruvate were mainly produced with a trace production of NO_2^- from NO_3^- (Fig. 7 and 8), suggesting that most reducing power was oxidized by atmospheric oxygen and only limited amount of reducing power was supplied to NO_3^- reduction (Fig. 9). The resultant pyruvate could be converted to acetate with production of reducing power, which can further be utilized by oxygen and the propionate pathway. *V. parvula* produced mainly pyruvate (Fig. 8), suggesting its low activity of the formate-acetate and the propionate pathways under aerobic conditions. According to the present study, *Veillonella* species can produce ATP through the aerobic lactate metabolism (Fig. 9); however, they are strictly anaerobes and cannot grow, maybe due to oxygen-labile systems independent from the lactate metabolic system.

The present study clearly showed that *Veillonella* species produce NO_2^- efficiently in the presence of lactate at a wide range of pH (neutral to acidic pH) under anaerobic conditions. It is well known that the environment in oral biofilm or some areas in the oral

cavity is anaerobic and becomes acidic and lactate-dominant after carbohydrate intake (Huang *et al*, 2011). The constant supply of NO_3^- from saliva and its intermittent supply from food such as green leafy vegetables support *Veillonella* species to produce NO_2^- and subsequently suppress other oral bacteria that can be associated with oral diseases such as caries. In this context, *Veillonella* species may play a balancing role in maintaining a health-associated oral microbiome by controlling the excessive activity of metabolism and growth of oral bacteria. Hence, consuming green leafy vegetables containing NO_3^- as daily intake; induce and enhance the NO_2^- production by oral *Veillonella*. Even though caries is a multi-factorial disease, some studies have already showed that consuming vegetables as dietary intake could reduce the severity of caries (Punitha *et al*., 2015).

In addition, after swallowing NO_2^- produced by *Veillonella*, NO_2^- enters the acidic stomach or contact with mammalian nitrite reductase where it is nonenzymatically and enzymatically metabolized, respectively to form bioactive nitrogen oxides such as nitric oxide (NO). Orally ingested NO_3^- clearly has robust NO-like effect systemically such as vasodilatory and lowering the blood pressure (Kapil *et al*., 2015, Montenegro *et al*., 2017). The cohabitation of nitrate-reducing bacteria such as *Veillonella* species in the oral cavity has a crucial effect to the general health, and it was reported by Montenegro *et al*., (2017) that elimination of oral nitrate-reducing bacteria by antiseptic reagent caused lowering the plasma nitrite with a concomitant increasing the blood pressure.

CONCLUSION

In conclusion, first, NO_2^- at low dose of 0.5 mM inhibited the growth of *Streptococcus mutans*, a representative caries-associated microorganism with a high acidogenicity and aciduricity, while it needed higher dose of 20 mM to inhibit the growth of *Veillonella* species, representative of oral NO_2^- producing bacteria. Second, NO_3^- and NO_2^- stimulated the growth of *Veillonella* species by shortening the lag phase of growth. Third, environmental factors (lactate, acidic pH, and anaerobic conditions) and growth conditions (the presence of NO_3^- or NO_2^-) increased the NO_2^- production of *Veillonella* species. Fourth, the NO_2^- production was linked to the lactate metabolism under anaerobic conditions, in which NO_3^- is reduced to NO_2^- by accepting the reducing power derived from the oxidization of lactate.

These findings strongly suggest that the consideration of daily intake of NO_3^- is crucial in maintaining our health conditions. Constant supply of NO_3^- from saliva and its intermittent supply from green leafy vegetables might alter the oral health condition by the promotion of the growth, metabolism of lactate to weaker acids, and the NO_2^- production of oral *Veillonella*. Subsequently, NO_2^- can suppress the other oral bacteria associated with oral disease such as caries, then enter the circulation and convert into NO by mammalian nitrite reductase or acidic stomach environment, resulting vasodilatation and lowering the blood pressure. This mechanism, the biochemical linkage between NO_3^-/NO_2^- and lactate metabolism in *Veillonella* species, may explain how oral *Veillonella* can maintain the oral and general health.

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FIGURE LEGENDS

Figure 1:

Effects of NO_3^- on the bacterial growth. The OD values of bacterial growth for 24 hours were indicated.

Values of average ± standard deviation were shown (N = 3 : *Streptococcus mutans*, *Veillonella atypica* and *Veillonella parvula*).

*Significant difference ($p<0.05$) in *Streptococcus mutans*, from control (without NO_3^-) (dunnettt's test).

Figure 2:

Effects of NO_2^- on the bacterial growth. The OD values of bacterial growth for 24 hours were indicated.

Values of average ± standard deviation were shown (N = 4 : *Streptococcus mutans*, N = 3 :*Veillonella atypica* and *Veillonella parvula*).

**Significant difference ($p<0.01$) in *Streptococcus mutans*, $^{\#\#}$ significant difference ($p<0.01$) in *Veillonella atypica*, and †† significant difference ($p<0.01$) in *Veillonella parvula* from control (without NO_2^-) (dunnettt's test).

Figure 3:

Bacterial response to NO_3^- or NO_2^- during growth of *Veillonella atypica* pre-cultured without KNO_3 or KNO_2 (a) , with KNO_3 (b) and with KNO_2 (c).

Values of average ± standard deviation were shown (N = 3).

*Significant difference (*p<0.05, **p<0.01) between no addition and KNO_3 $^{\#}$ significant difference ($^{\#}$p<0.05, $^{\#\#}$p<0.01) between no addition and KNO_2, and † significant difference (†p<0.05, ††p<0.01) between KNO_3 and KNO_2 (tukey's test).

Figure 4:

Bacterial response to NO_3^- or NO_2^- during growth of *Veillonella parvula* pre-cultured (a) without KNO_3 or KNO_2 (a) , with KNO_3 (b) and with KNO_2 (c).

Values of average ± standard deviation were shown (N = 3).

*Significant difference (*p<0.05, **p<0.01) between no addition and KNO_3 $^{\#}$ significant difference ($^{\#}$p<0.05, $^{\#\#}$p<0.01) between no addition and KNO_2, and † significant difference (†p<0.05, ††p<0.01) between KNO_3 and KNO_2 (tukey's test).

Figure 5:

NO_2^- production from NO_3^- by the resting cells of *Veillonella atypica* in aerobic condition at pH 7 (a) and pH 5 (b), and in anaerobic condition at pH 7 (c) and pH 5 (d).

Values of average ± standard deviation were shown (N = 3 : aerobic condition and anaerobic condition).

*Significant differences (*p<0.05, **p<0.01) comparing the groups between bacterial intact cell grown only with TYL (without KNO_3 or KNO_2), KNO_3 or KNO_2 (tukey's test).

Significantly differences (#p<0.05, ## p<0.01) comparing the groups in the same grown condition with 0 mM of lactate (dunnett's test).

Figure 6:

NO_2^- production from NO_3^- by the resting cells of *Veillonella parvula* in aerobic condition at pH 7 (a) and pH 5 (b), and in anaerobic condition at pH 7 (c) and pH 5 (d).

Values of average ± standard deviation were shown (N = 3: aerobic condition and anaerobic condition).

Significant differences ($p<0.05$, **$p<0.01$) comparing the groups between bacterial intact cell grown only with TYL (without KNO_3 or KNO_2), KNO_3 or KNO_2 (tukey's test).

Significantly differences (#$p<0.05$, ## $p<0.01$) comparing the groups in the same grown condition with 0 mM of lactate (dunnett's test).

Figure 7:

Metabolic end products from lactate during the NO_2^- production by the resting cells of *Veillonella atypica*.

Values of average ± standard deviation were shown (N = 4).

Figure 8:

Metabolic end products from lactate during the NO_2^- production by the resting cells of *Veillonella parvula*.

Values of average ± standard deviation were shown (N = 3).

Figure 9:

Proposed pathways for NO_3^- and lactate metabolism of *Veillonella* species.

Fig. 1

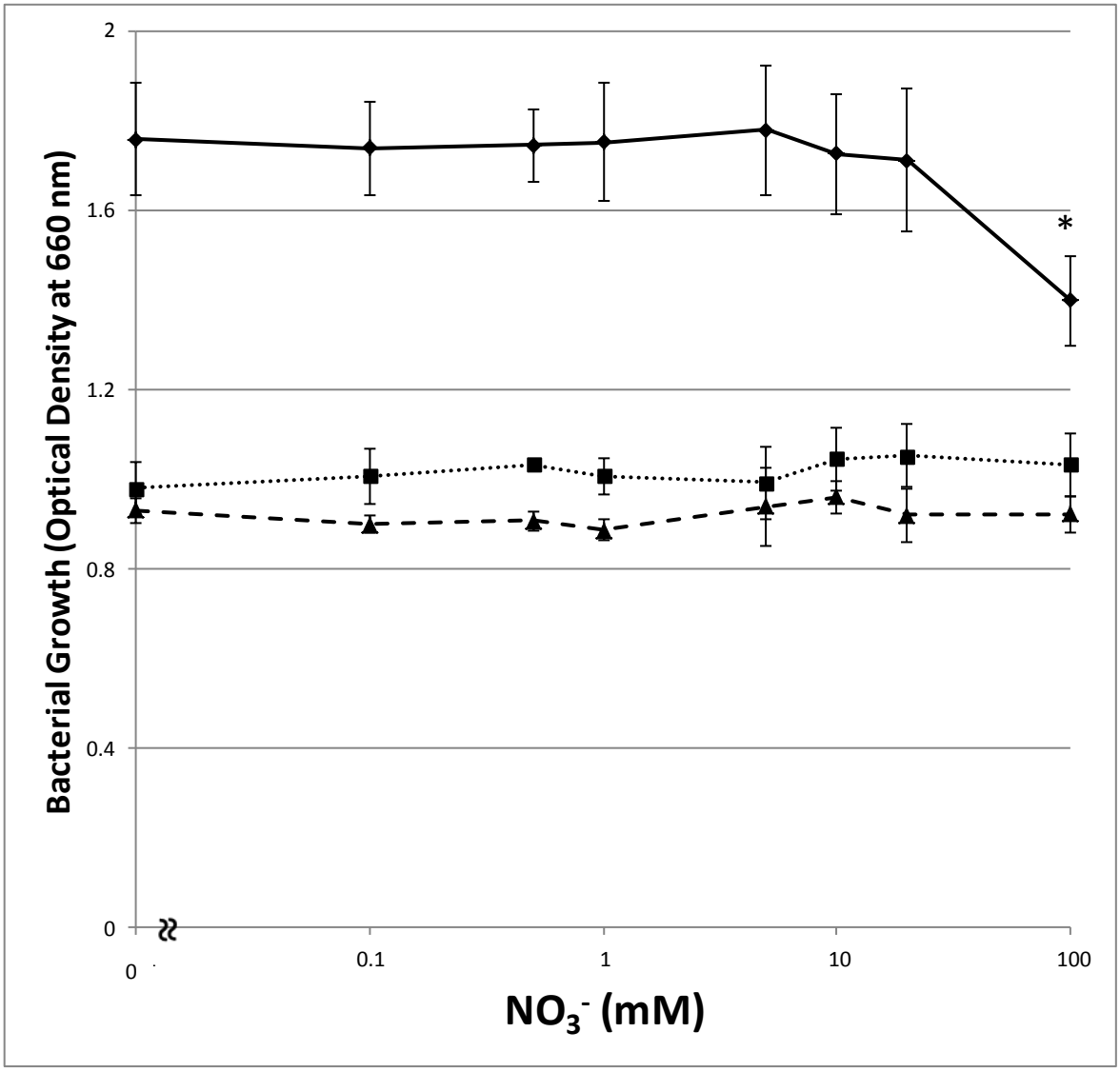

Bacterial Growth (Optical Density at 660 nm)
2
1.6
1.2
0.8
0.4
0
*
0
0.1
1
10
100
NO3- (mM)
Streptococcus mutans
Veillonella atypica
Veillonella parvula

Fig 2.

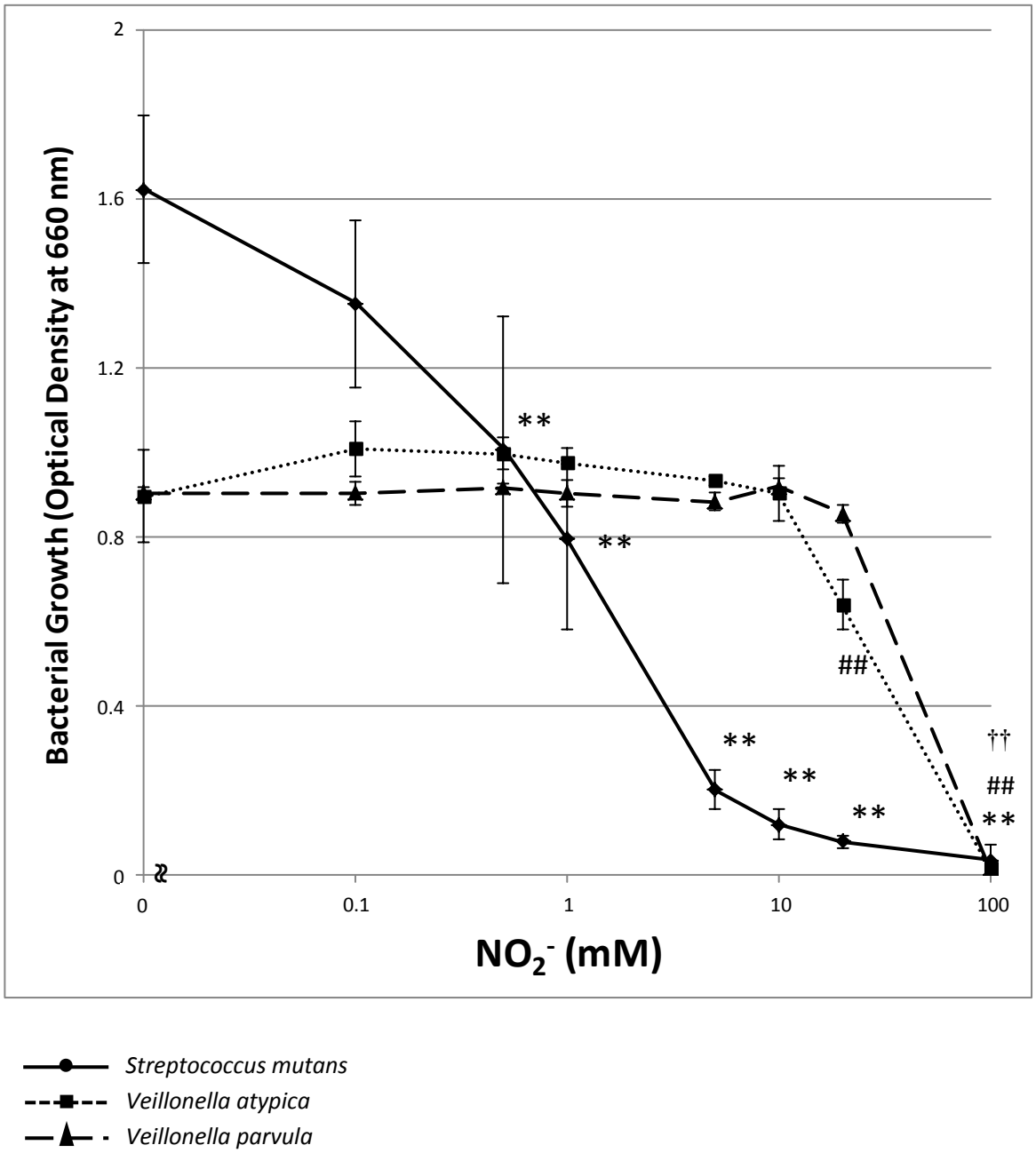

Bacterial Growth (Optical Density at 660 nm)
2
1.6
1.2
0.8
0.4
0
0
0.1
1
10
100
NO2- (mM)
**
**
**
**
**
##
##
††
Streptococcus mutans
Veillonella atypica
Veillonella parvula

Fig 3.

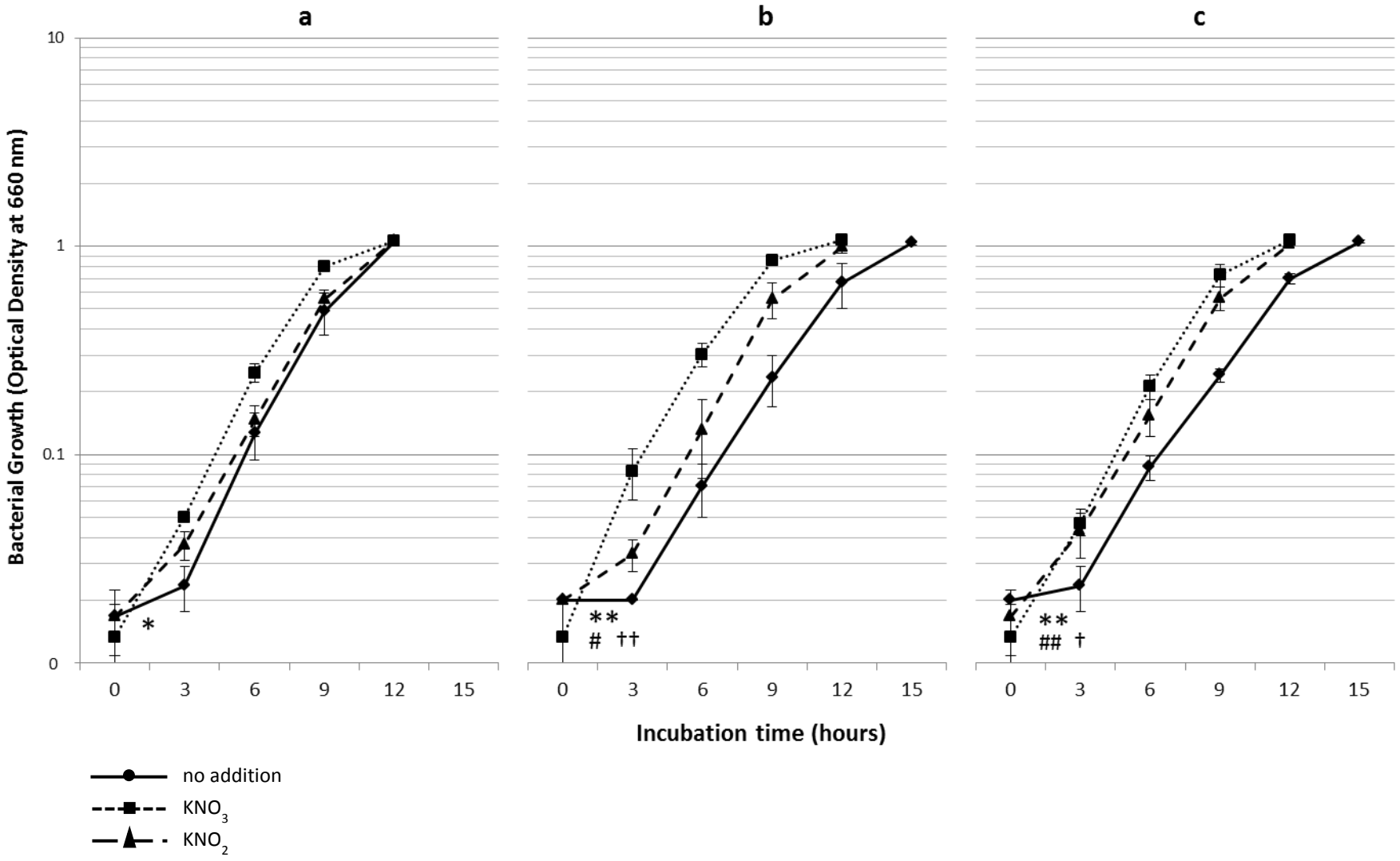

a
b
c
10
1
0.1
0
Bacterial Growth (Optical Density at 660 nm)
0
3
6
9
12
15
Incubation time (hours)
*
**
#
††
**
##
†
no addition
KNO3
KNO2

Fig 4.

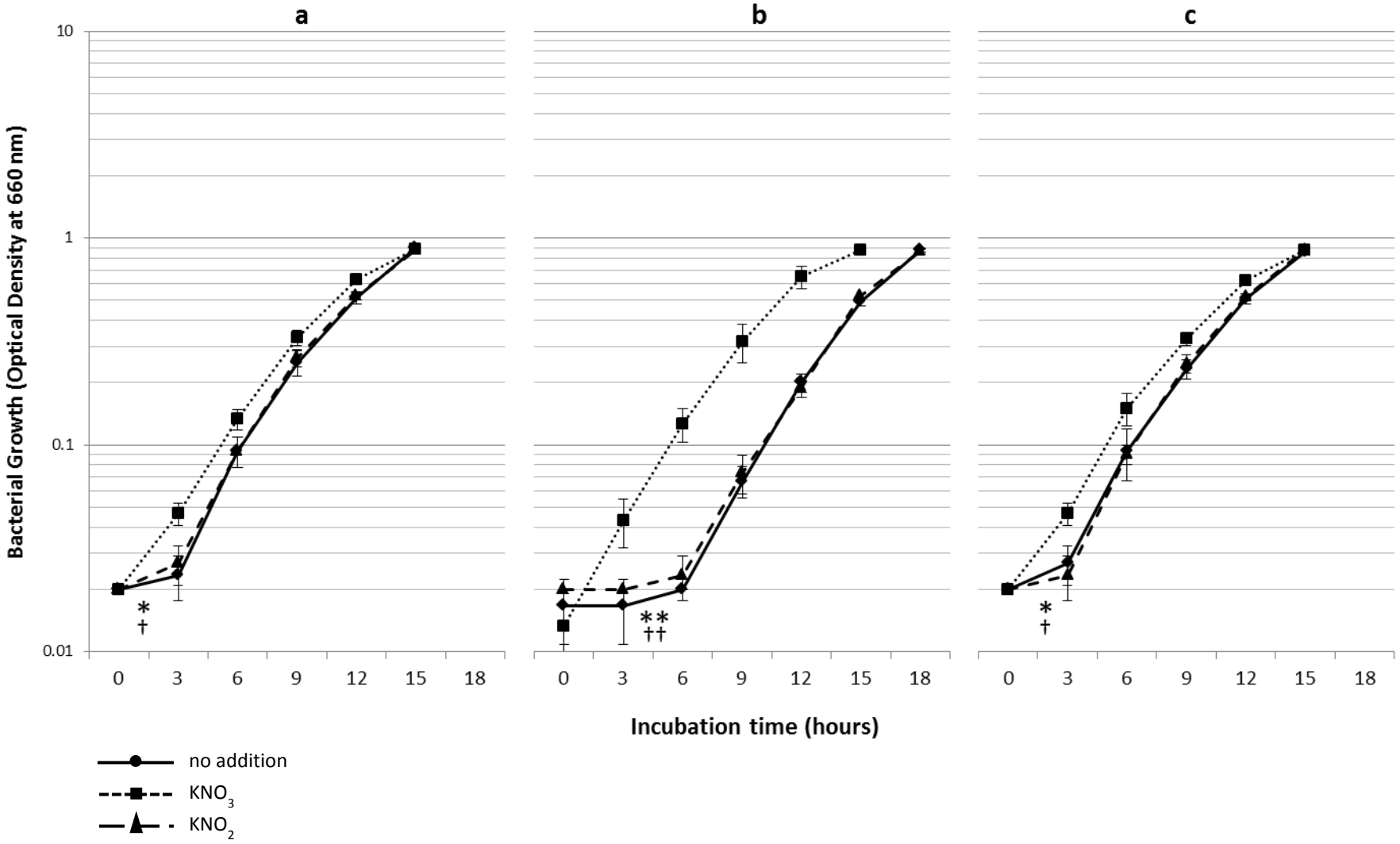

a
b
c
10
1
0.1
0.01
Bacterial Growth (Optical Density at 660 nm)
0
3
6
9
12
15
18
Incubation time (hours)
*
†
**
††
no addition
KNO3
KNO2

Fig 5.

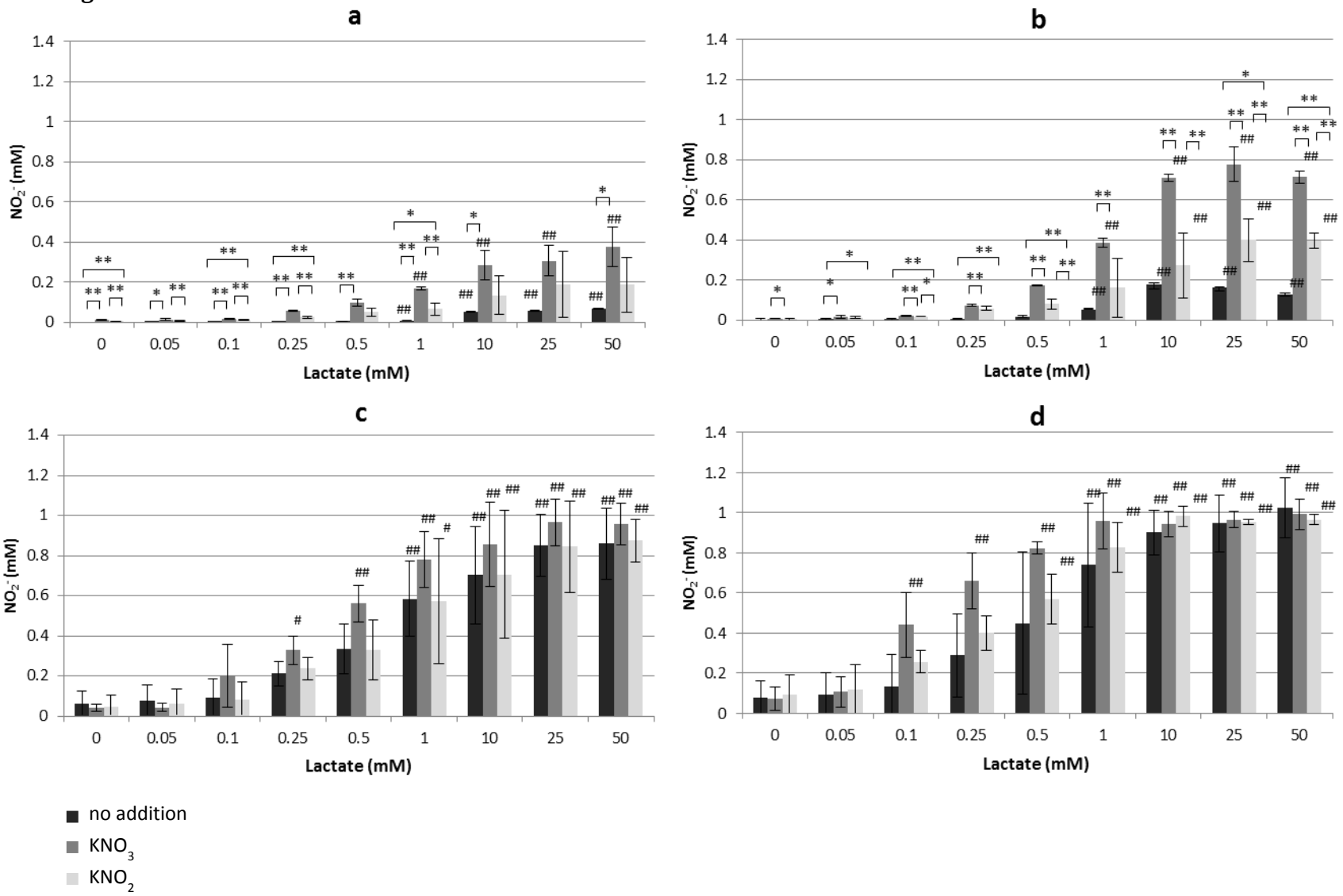

a
b
c
d
NO_2^- (mM)
Lactate (mM)
0
0.05
0.1
0.25
0.5
1
10
25
50
no addition
KNO_3
KNO_2

Fig 6.

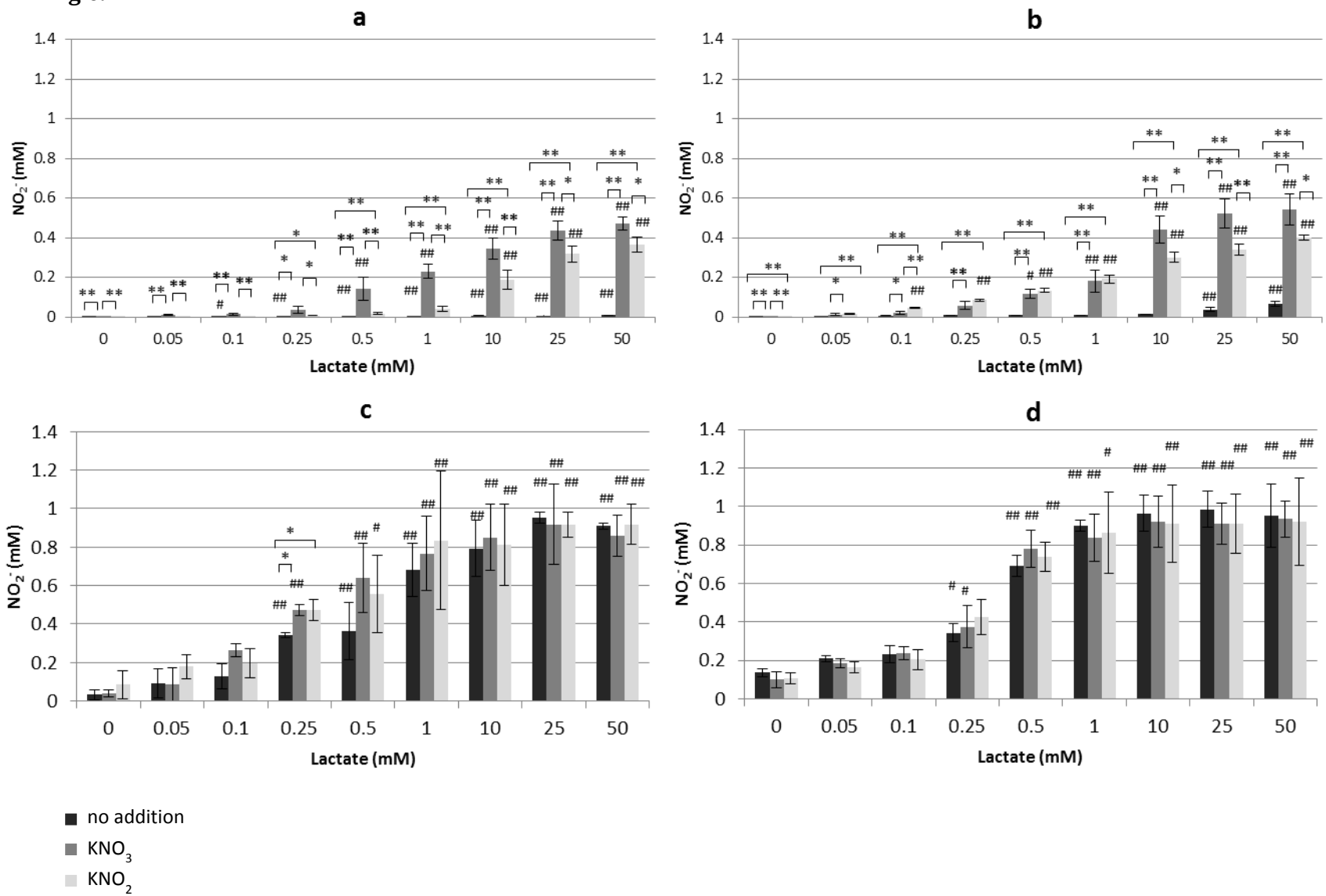

a
b
c
d
NO_2^- (mM)
Lactate (mM)
0
0.05
0.1
0.25
0.5
1
10
25
50
no addition
KNO_3
KNO_2

Fig 7.

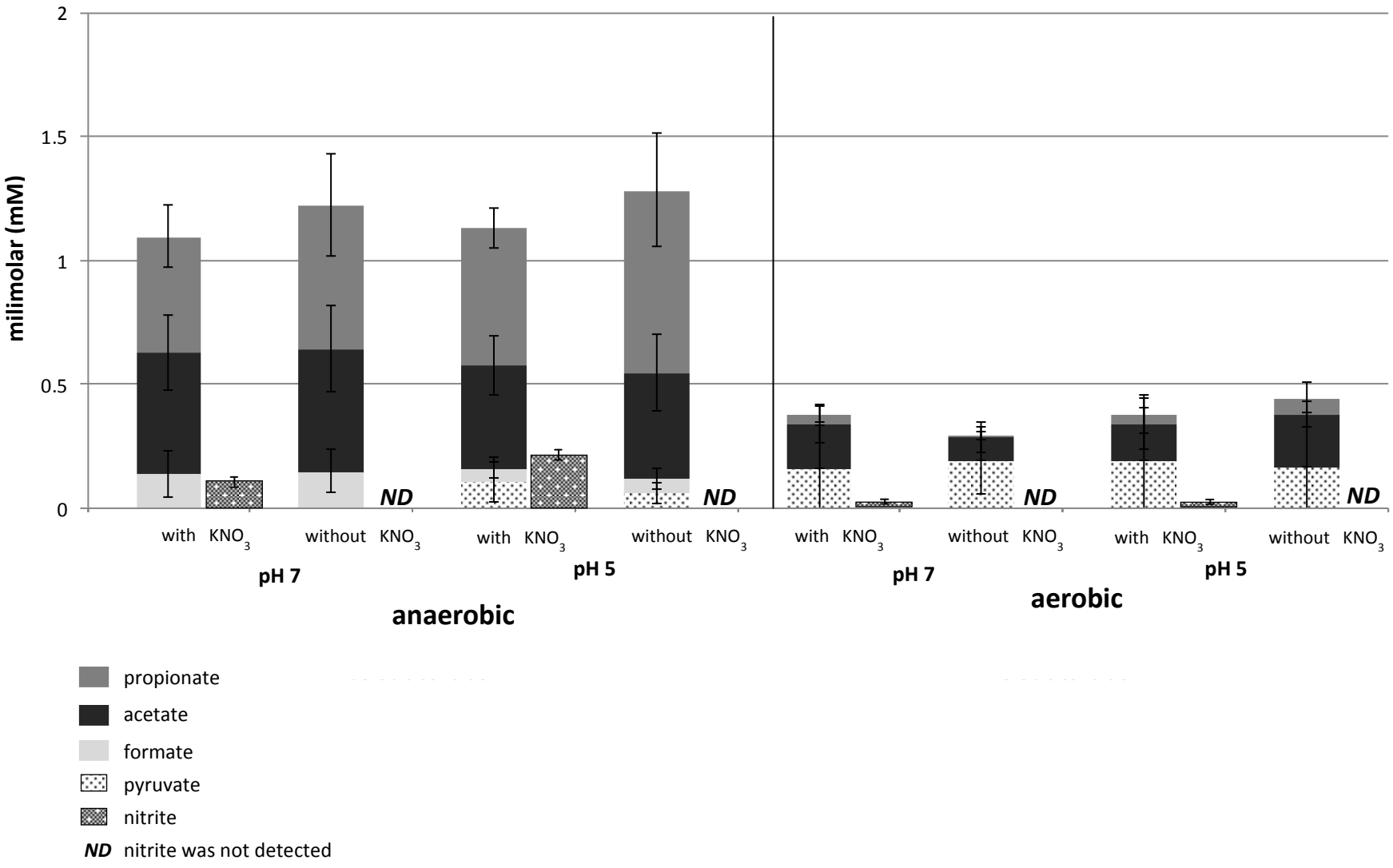

2
1.5
1
0.5
0
milimolar (mM)
ND
ND
ND
ND
with KNO3
without KNO3
with KNO3
without KNO3
with KNO3
without KNO3
with KNO3
without KNO3
pH 7
pH 5
pH 7
pH 5
anaerobic
aerobic
propionate
acetate
formate
pyruvate
nitrite
ND nitrite was not detected

Fig. 8.

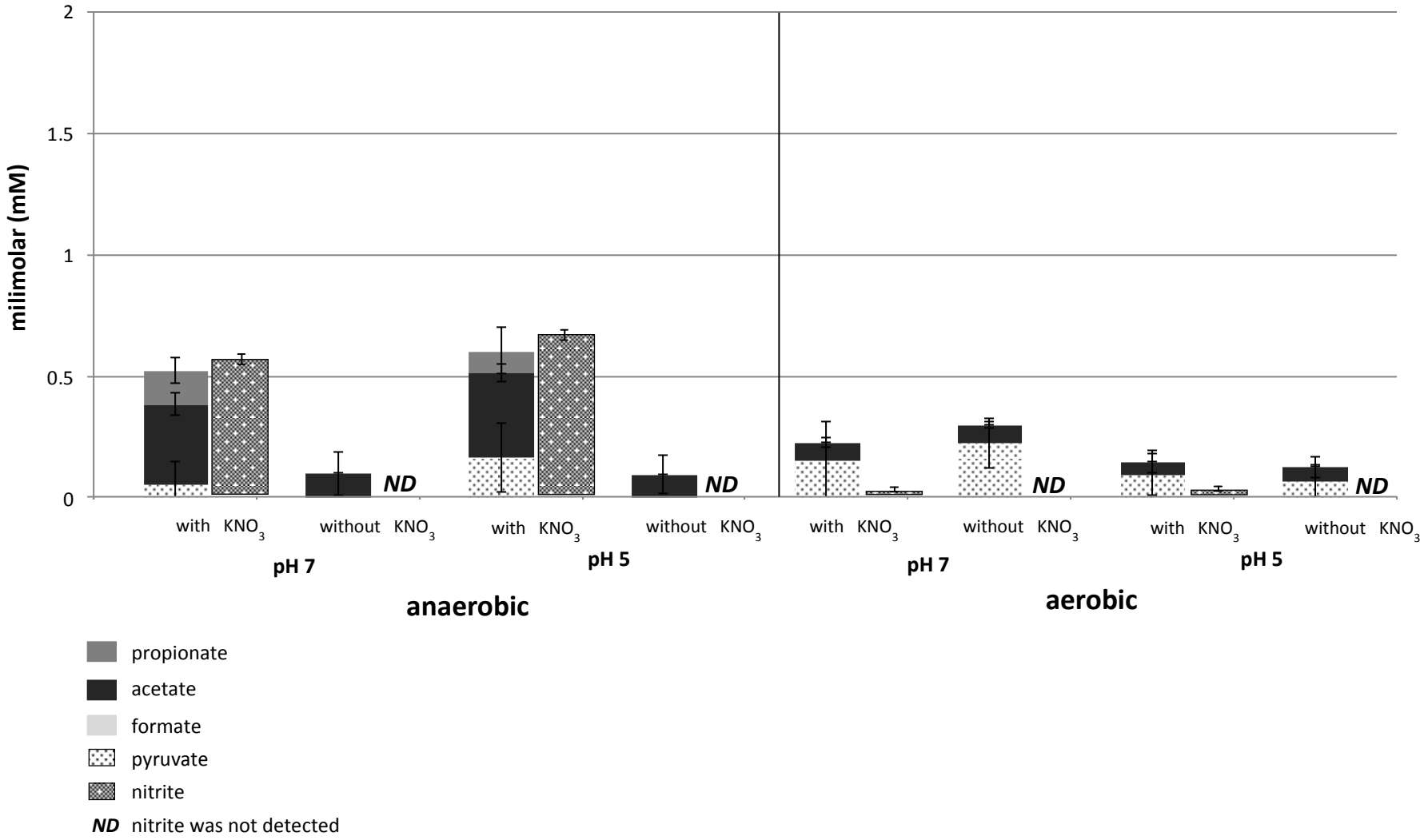
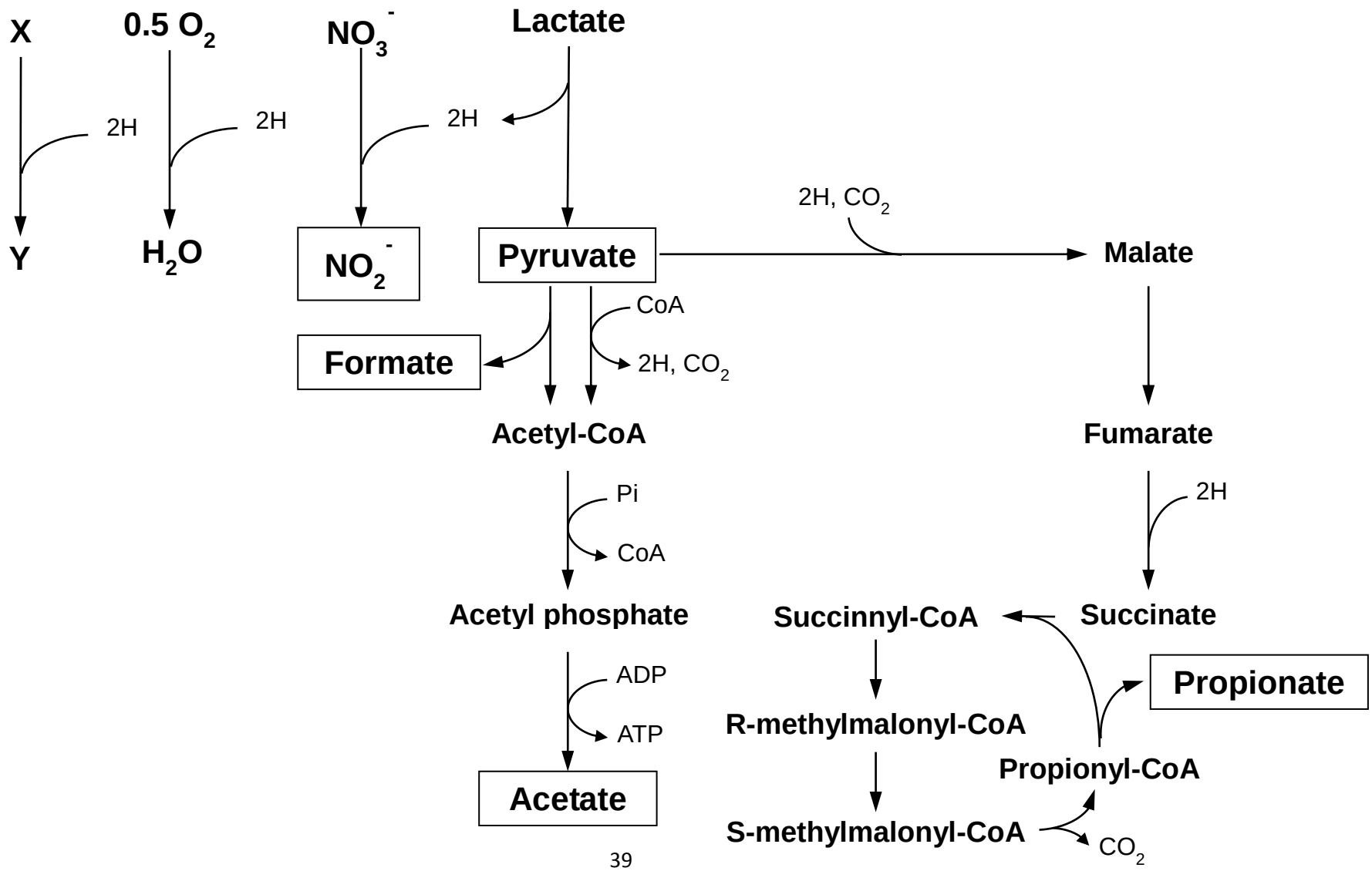

2
1.5
1
0.5
0
milimolar (mM)
with KNO3
without KNO3
with KNO3
without KNO3
with KNO3
without KNO3
with KNO3
without KNO3
ND
ND
ND
ND
pH 7
pH 5
pH 7
pH 5
anaerobic
aerobic
propionate
acetate
formate
pyruvate
nitrite
ND nitrite was not detected

Fig 9.


X
0.5 O2
NO3-
Lactate
2H
2H
2H
Y
H2O
NO2-
Pyruvate
2H, CO2
Malate
CoA
Formate
2H, CO2
Acetyl-CoA
Fumarate
Pi
2H
CoA
Acetyl phosphate
Succinnyl-CoA
Succinate
ADP
Propionate
R-methylmalonyl-CoA
ATP
Propionyl-CoA
Acetate
S-methylmalonyl-CoA
CO2